Ca^{2+}/Calmodulin-Dependent Protein Kinase II-Dependent Regulation of TREK-1

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Abstract

Ion channels serve as core constituents of macromolecular complexes that regulate cell membrane excitability and cell signaling pathways. Thus, proper ion channel function is essential for heart function. Importantly, ion channel activity is heavily regulated by posttranslational modifications. The mechano-sensitive two pore K^+ channel TREK-1 has been identified as an important channel for proper repolarization in the heart and has been shown to induce actin reorganization resulting in filopodia-like protrusions in COS M6 cells. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), an important multifunctional serine/threonine kinase that has been linked to cardiac diseases such as hypertrophy, arrhythmias, and cardiac failure, has been identified as a potential regulator of TREK-1 activity. However, little is known about the mechanism and/or role of CaMKII-dependent regulation of TREK-1. We created a library of TREK-1 mutants using site-directed mutagenesis to ablate putative CaMKII phosphorylation sites located in the intracellular domains of the channel. Wildtype (WT) and mutant TREK-1 constructs were engineered into a GFP-expressing pIRES vector and expressed in COS M6 cells. Cells were immunostained with TREK-1 antibody and the cell shape, size, and structure were analyzed using confocal microscopy. In parallel, TREK-1 fragments corresponding to the major intracellular domains (N-terminal, linker, and C-terminal) were generated as GST fusion proteins and used for in vitro CaMKII phosphorylation assays. Our studies indicate that CaMKII phosphorylates TREK-1 at a site in the N-terminal intracellular domain. Cells expressing the TREK-1 N-terminal mutant (Ser46Ala) showed little difference in cytoskeletal organization compared to WT-expressing cells. However, these cells were significantly smaller than both untransfected and WT cells. These studies suggest that CaMKII phosphorylates TREK-1 at a site in the N-terminus and that although this site may not play a significant role in the formation of membrane protrusions, this site may be part of cell signaling pathways that affect cell size. Future studies will determine whether CaMKII

targets additional sites on the channel and whether CaMKII-dependent phosphorylation of TREK-1 is important for regulating other cell functions.

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1. Introduction

1.1 Background Information

Proper ion channel function is essential to human health. Ion channels serve as core constituents of well-defined macromolecular complexes that regulate cell membrane excitability and cell signaling pathways. Importantly, ion channels are heavily regulated by posttranslational modification (e.g. phosphorylation, oxidation), which can have a wide array of effects on how ion channels behave.

One important ion channel that has been increasingly studied due to its importance in excitable cells is the K_{2P} channel TREK-1 (Figure 1).[1,2] The TREK-1 channel is expressed in the brain,[3,4,5] dorsal root ganglia,[6,7] smooth muscles surrounding the prostate and gastrointestinal tract,[4,5] cardiovascular system,[8,9] and myocardium.[10,11] TREK-1 is an important channel due to its role in the biological response to anesthesia,[12] neuroprotection,[13] sensitivity to pain and temperature,[6,7] depression,[14] epilepsy,[15] and architecture of the anterior eye chamber.[16] At the cellular level, membrane excitability is regulated by TREK-1 and has been shown to be important for proper membrane repolarization. In cardiomyocytes, it has been shown that TREK-1 is part of a macromolecular complex with the actin-associated polypeptide β_{IV}-spectrin, which regulates TREK-1 localization to the plasma membrane (Figure 2). Localization due to β_{IV}-spectrin is essential for proper channel activity and repolarization.[11] TREK-1 activity is also dependent upon a large range of chemical and physical stimuli and is activated by mechanical stretching,[17] intracellular acidosis,[18] increased temperature,[19] polyunsaturated fatty acids,[13] and volatile and gaseous anesthesia.[20]

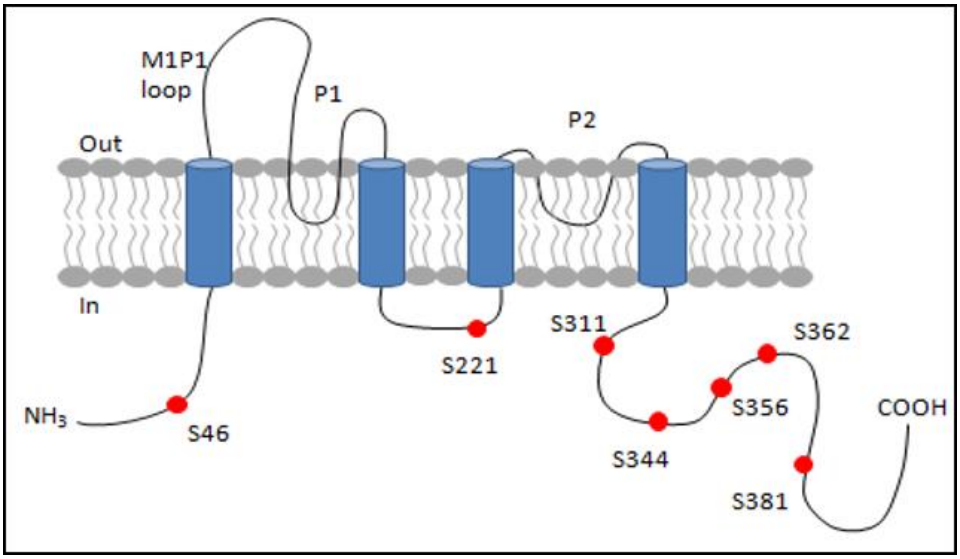

Figure 1: TREK-1 structure with putative CaMKII phosphorylation sites (Arg-x-x-Ser/Thr) in the intracellular domains of TREK-1 indicated by red dots.

In addition to its roles in membrane excitability, TREK-1 has been linked to cytoskeletal restructuring through its interaction with actin.[21] Actin is an essential structural protein that can be present as both a free monomer in the form of G-actin and a linear polymer microfilament called F-actin. This protein is generally the most abundant protein in eukaryotic cells and is important for cell motility, maintenance of cell shape and size, mechanical properties of the cell, cellular signaling pathways, protein localization, and other essential cell functions.[22] Inhibition of TREK-1 expression has been shown to reduce F-actin and increase deformability in alveolar epithelial cells.[23] Additionally, TREK-1 expression has been linked to the production of actin-filled filopodia-like structures.[21] Although there is strong evidence that TREK-1 expression regulates cytoskeletal restructuring by altering actin, the mechanism behind this is not well understood. Additionally, much is still unknown as to how this altered actin patterning affects cell size, structure, mechanical properties, excitability, and function.

Posttranslational modification of TREK-1 has been shown to be extremely important for regulating its role in membrane excitability and cytoskeletal reorganization. TREK-1 has two important phosphorylation sites at Ser311 and Ser344 (human TREK-1). Activation of protein kinase A (PKA) and protein kinase C (PKC) via a G-coupled protein channel signaling pathway leads to phosphorylation of S344 and S311 sequentially to inhibit ionic current through TREK-1.[24] In addition to regulating the channel conductance, S344 regulates signaling between TREK-1 and actin altering the cytoskeleton of the cells. Phosphorylation of TREK-1 at this amino acid highly increases the percentage of cells exhibiting filopodia-like protrusions.[21] Although phosphorylation at these sites have been studied, other potential phosphorylation sites of TREK-1 and their effects on cell excitability and cytoskeletal reorganization remain unknown.

An important protein that regulates a variety of intracellular proteins via phosphorylation is Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). CaMKII is a multifunctional serine/threonine kinase that has four different isoforms that show varied tissue distribution. CaMKII regulates a wide array of cellular functions including ion channel activity,[25,26] organelle transport,[27] metabolism,[28] transcription,[29] and cytoskeletal structure.[30] Hormone secretion,[31] cardiac excitation-contraction coupling,[26] and synaptic plasticity[32] are all modulated by CaMKII and abnormal CaMKII behavior has been linked to cardiac arrhythmias,[33] hypertrophy,[34,35,36] heart failure,[34,37] and cognitive defects.[38]

Activity of CaMKII depends upon intracellular Ca^{2+} binding to calmodulin which in turn binds to and inhibits the regulatory domain of CaMKII.[39] Posttranslational modification of CaMKII by autophosphorylation of Thr287,[40] oxidation of Met281/282,[41] O-linked glycosylation of Ser280,[42] or NO-dependent nitrosylation of Cys116, Cys273, or Cys290 also induce CaMKII activity,[43] while phosphorylation of Thr306/307 reduce CaMKII activity.[40] Due to these posttranslational modifications, CaMKII activity is usually upregulated during stress in tissues which has been linked to different pathologies.[4235,42]

Like TREK-1, CaMKII is a part of a macromolecular complex with β_{IV}-spectrin (Figure 2). Previous studies have shown that the CaMKII/β_{IV}-spectrin signaling complex is essential for membrane excitability. B_{IV}-spectrin targets CaMKII to membrane proteins at the intercalcated disc in cardiomyocytes and the axon initial segment (AIS) in neurons for regulation. Phosphorylation of $Na_v1.5$ by CaMKII is critical for proper heart function.[44] However, little is still known about this signaling complex and how it affects other membrane proteins and cell characteristics, like cell structure.

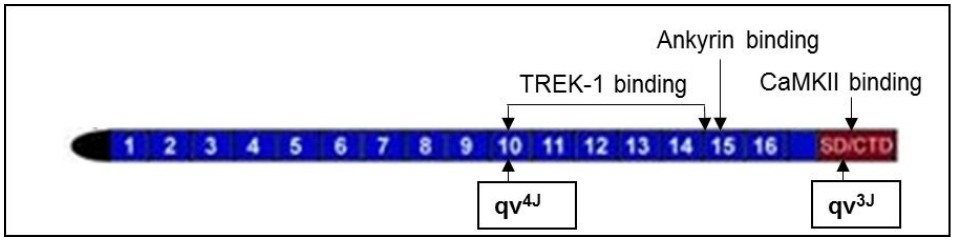

Figure 2: Structure of β_{IV}-spectrin, its major binding partners, and two mutant mice strains that exhibit truncation of β_{IV}-spectrin.

1.2 Objectives

Our overall objective is to understand the pathways regulating electrical and structural remodeling of the myocardium in response to stress. The main objective of this study was to determine the molecular mechanism underlying TREK-1-dependent regulation of cellular remodeling. We hypothesize that CaMKII dependent phosphorylation of TREK-1 regulates channel activity and/or cytoskeletal restructuring. In order to increase our understanding of the roles of TREK-1 and CaMKII in cytoskeletal restructuring, the following two aims were investigated.

<u>Specific Aim 1</u>: Determine the mechanism of CaMKII phosphorylation of TREK-1.

We hypothesized that CaMKII directly phosphorylates TREK-1 to regulate its activity. In order to determine the site of phosphorylation of TREK-1 by CaMKII, a phosphorylation assay was carried out with CaMKII and the three intracellular domains of TREK-1 which were generated as GST-fusion proteins.

<u>Specific Aim 2</u>: Determine the effects of phosphorylation at the putative CaMKII phosphorylation site S46 on cell structure.

We hypothesize that CaMKII-dependent phosphorylation of TREK-1 regulates cellular remodeling. In order to test this hypothesis, we mutated Ser at 46 position to Ala (S46A). Wildtype and mutant (Ser46Ala) TREK-1 were transfected into HEK cells and various properties of cell structure (percentage of cells with protrusion, number of protrusions, cell circumference, cell 2D-surface area, and the circumference to 2D-surface area ratio) were analyzed.

1.3 Significance

This study addresses an important question of how TREK-1 and posttranslational modification of TREK-1 via CaMKII modulates cell structure. There is currently a large gap in our understanding of how TREK-1 interacts with actin and how this interaction affects organisms at the cellular, tissue, and organ levels. Specifically, this research could shed new light on myocardial diseases like such as hypertrophy, arrhythmias, and heart failure. Linking CaMKII to TREK-1 and cellular structural changes would help further our understanding of CaMKII in these cardiac diseases and potentially lead to novel therapies. Additionally this research could help us better understand TREK-1's roles in the biological response to anesthesia, neuroprotection, sensitivity to pain and temperature, depression, epilepsy, and architecture of the anterior eye chamber.

2. Methods and Materials

2.1 Cloning

KCNK2 gene (TREK-1) was purchased from commercial vendor (Open Biosystems) and engineered into the pIRES vector (Invitrogen). Putative CaMKII binding sites were identified by screening the amino acid sequence of TREK-1 for the 4-amino acid CaMKII binding motif Arg-x-x-Ser/Thr. Forward and reverse primers (Integrated DNA Technologies) for each of the putative phosphorylation sites were designed to mutate the serine residues to alanine residues. Site-directed mutagenesis was performed using QuikChange II (Agilent) and constructs were confirmed by sequencing.

2.2 Western Blot Analysis

Wild type TREK-1 and TREK-1 mutants were transfected into HEK cells and the cell lysates (protein concentration normalized by standard BCA protocols) were analysed by SDS—Page and immunoblotting. Small differences in protein loading were corrected using normalization with GAPDH. The following anitbodies were used for immunoblotting and/or immunostaining: TREK-1 (Santa Cruz)(reference 21 for b4 paper) and GAPDH (Fitzgerald).

2.3 Phosphorylation Assay

PCR primers were designed to TOPO-clone the N-terminal, M2-M3 Linker, and C-terminal regions of TREK-1 based on a sequence obtained from GeneBank. To generate constructs for GST- fusion protein expression and the phosphorylation assay, cDNAs were were engineered in-frame into pGEX6P1 (GE Healthcare). Vectors were completely sequenced to confirm the sequence identity. Bacterial cells expressing the GST-fusion constructs were lysed in the presence of GST-beads. Activated CaMKII (10

μL) and the GST-beads with bound GST-TREK-1 fusion proteins were incubated for 15 minutes in a reaction solution (30 μL tris binding buffer, 80 μg CaM, 10 μL of 20 mM $CaCl_2$, 2 μL p32 ATP, and 10 μL MgCl/ATP). The solution was washed thoroughly six times using wash buffer (1X PBS and 5 mM EDTA) and analyzed on a gel and subjected to radiography.

2.4 Mammalian Cell Culture

HEK and COS M6 cells were cultured in a growth medium (Dulbecco's Modified Eagle Medium (Gibco), 10% PBS, 1%Penicillin, 1%Streptomyocin). For western blot analysis, HEK cells were plated at 50% confluency and allowed to grow for 48 hours after transfection using a Quiagen transfection kit.

2.5 Immunostaining and Confocal Microscopy

For imaging, COS M6 cells were plated on MatTek slides at 25% confluence and allowed to grow for 24 hours before cell transfection. Transfection was accomplished by using a Quiagen transfection kit. Cells were allowed to grow for 24 hours after transfection and were fixed in 2% PFA. Cells were blocked in a PBS blocking buffer (0.15% Triton X-100 and 3% BSA) and incubated in primary antibody for 2 hours at room temperature. Cells were washed 5X with blocking buffer and then incubated in secondary antibody (Alexa-Fluor-568) for 2 hours at room temperature. Cells were washed with blocking buffer 5X and mounted using Vectashield with DAPI (Vector). Images were collected on a Zeiss 780 confocal microscope [objective W Plan Apochromat 40X/1.0 DIC (Zeiss), pinhole equals 1.0 Airy Disc] using the Carl Zeiss Imaging software.

2.6 ImageJ Analysis

Images obtained through confocal microscopy were analyzed using ImageJ software to define the circumference and 2D-area of the cells. The Bioformats plug-in for ImageJ (Open Microscopy Environment) was used to open and analyze images. The default over/under threshold was adjusted until the cell boundaries were within the limits. The area within the boundary and circumference of the boundary were calculated by the ImageJ software.

2.7 Statistical Analysis

P-values were determined for comparison using unpaired Student's t-tests (two-tailed). P values were considered statistically significant if $P<.05$. Values are expressed as means±SEM.

3. Results

3.1 Phosphorylation Analysis of TREK-1 Mutant Library

In order to study the effect of TREK-1 posttranslational modifications on actin cytoskeleton, a library of TREK-1 mutants was created by mutating putative CaMKII phosphorylation sites (sites with amino acid sequence: Arg-X-X-Ser/Thr) in TREK-1 gene. Seven putative CaMKII binding sites in the TREK-1 gene (Ser 46, Ser 221, Ser 311, Ser 344, Ser 356, Ser 362, Ser 381) were identified and the Ser amino acids in these positions were mutated to Ala to prevent the phosphorylation by CaMKII. (Figure 1). These mutations were confirmed through DNA sequencing and their protein expression was confirmed through Western Blot Analysis (Figure 3). TREK-1 fragments corresponding to the major intracellular domains [N-terminus (AA 1-115 with one putative site), M2-M3 linker (AA 190-229), and C-terminus (AA 305-422)] were generated as GST fusion proteins and used for in vitro CaMKII phosphorylation assay as described in section 2.3. The results of the phosphorylation assay indicate that CaMKII phosphorylates the N-terminal region of TREK-1, where the Ser46 putative site is located (Figure 3).

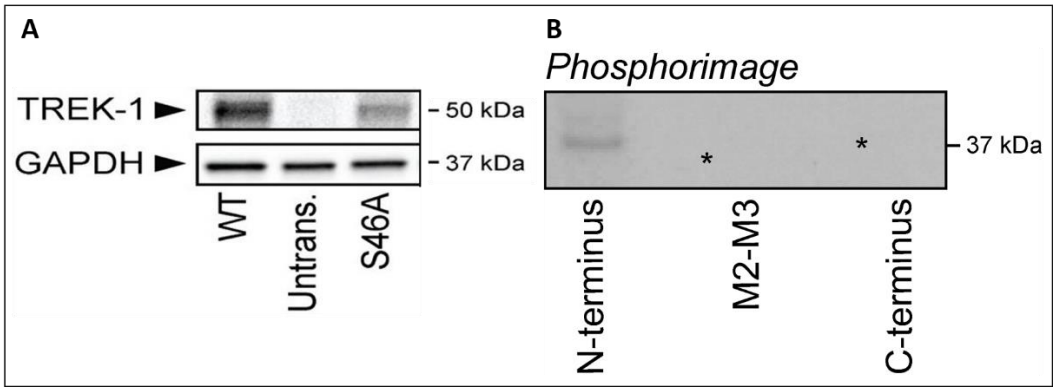

Figure 3: : (A) Western blot showing TREK-1 expression in HEK cells that were transfected with WT TREK-1, untransfected, and transfected with S46A mutant TREK-1. (B) CaMKII phosphorylation assay on intracellular domains of TREK-1. Asterisks indicate location of M2-M3 and C-terminus purified proteins.

3.2 TREK-1 expression promotes membrane projections

The identification of the TREK-1 N-terminus as a target for CaMKII phosphorylation led us to test the role of the S46 site in cytoskeletal reorganization. Untransfected COS M6 cells along with COS M6 cells transfected with WT and S46A mutant TREK-1 were imaged with confocal microscopy (Figure 4). In general, it is observed that both WT and mutant TREK-1 induce morphological changes in the cell structure. TREK-1 appears to be localized throughout the cell membrane but is concentrated in the filopodia-like protrusions. Although these protrusions were observed in all three cell types, they were more frequently observed in cells expressing WT TREK, than in un-transfected cells. Cells expressing S46A TREK-1 showed greater tendency to display protrusions than un-transfected cells, but the percentage of cells which displayed protrusions was smaller in number compared to cells with WT TREK (Figure 5). Along with the percentage of cells that exhibited filopodia-like protrusions, the number of protrusions per cell was used to distinguish the role of TREK in the production of these protrusions. A similar trend is observed for the number of protrusions per cell, where the

expression of WT TREK-1 appears to increase the number of protrusions per cell in comparison with untransfected cells. S46A TREK expressing cells have greater protrusions per cell than untransfected cells, but exhibit fewer protrusions on average than cells with WT TREK (Figure 6).

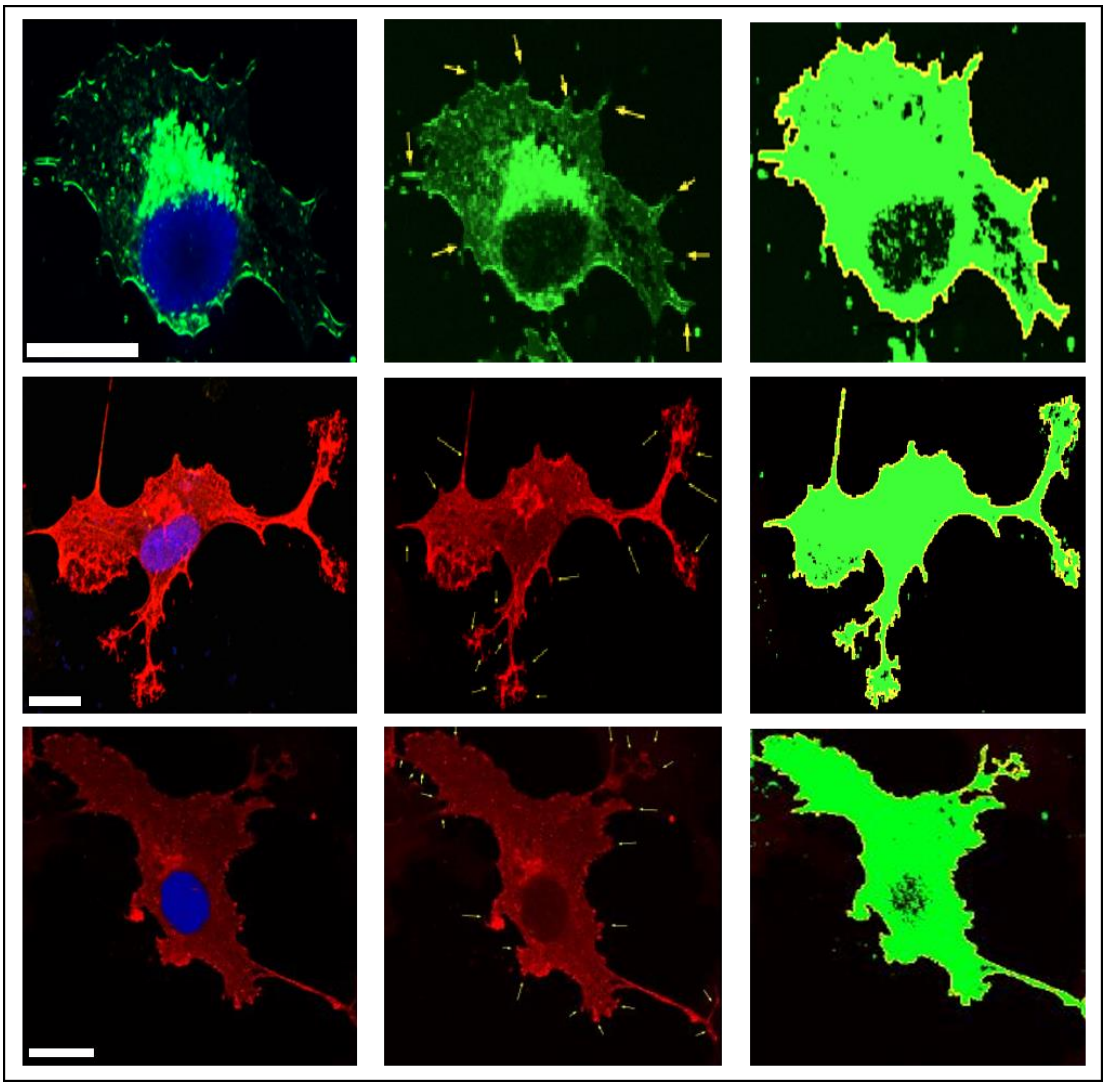

Figure 4: (A) Untransfected COS M6 cells were stained with Di-8 ANEPPS (green). COS M6 cells were transfected with **(B)** GFP-expressing WT and **(C)** S46A constructs immunostained for TREK-1 (red) and nuclei were stained DAPI (blue). Arrows represent identified projections mapped using ImageJ. The third column indicates the areas and circumferences found using ImageJ software.

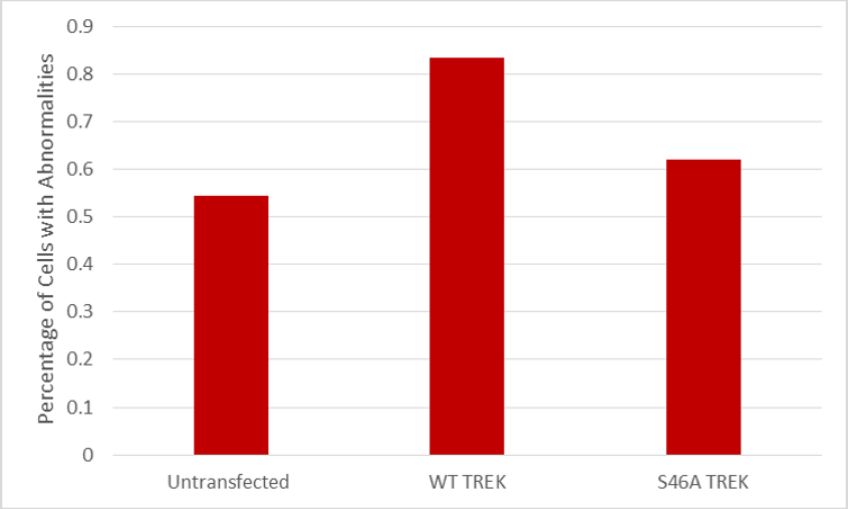

Figure 5: Percentage of cells with significant projections for untransfected, wild type (WT) and S46A mutant transfected COS M6 cells.

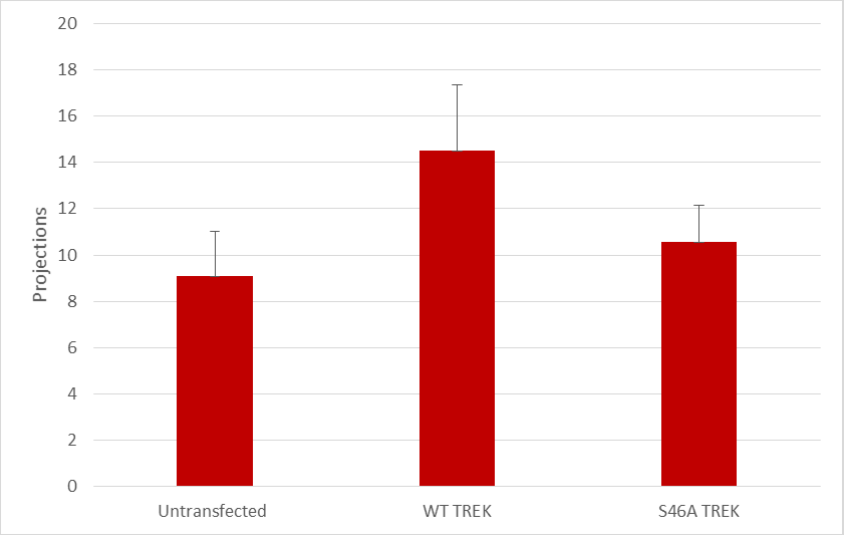

Figure 6: Number of projections for untransfected, wild type (WT) and S46A mutant transfected COS M6 cells. P=NS.

3.3 S46A expressing cells have altered size

The untransfected, WT, and mutant TREK-1 cell images were analyzed with ImageJ software to determine the circumference of the monolayer of cells and their 2D-area. Interestingly, it was observed that S46A mutant cells were significantly smaller than both, un-transfected cells and cells expressing WT TREK-1. The circumference of the untransfected and WT cells were nearly identical, while the circumference of the S46A mutants was about 65% of the other two groups (Figure 7). Analysis of 2D-area shows that the WT TREK-1 cells were smaller than the untransfected cells, however, the S46A mutants were significantly smaller than either of the other to two groups. These two findings indicate that a lack of phosphorylation at this site seems to induce a decrease in cell size.

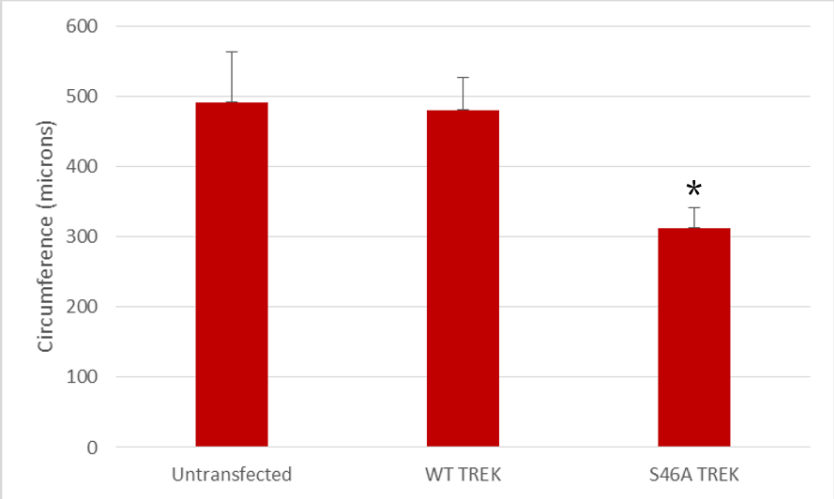

Figure 7: Circumference of untransfected, wild type (WT) and S46A mutant transfected COS M6 cells. P=0.0024

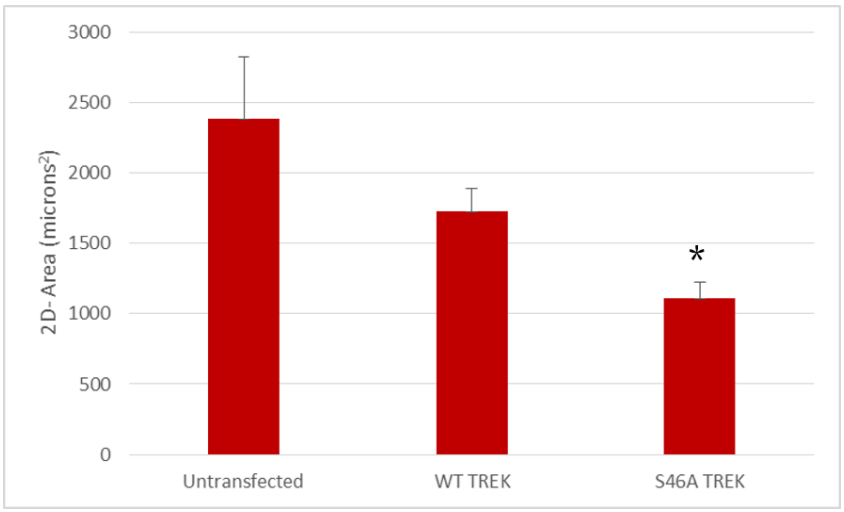

Figure 8: 2D-area of untransfected, wild type (WT) and S46A mutant transfected COS M6 cells. P= 0.0029

3.4 TREK-1 modifies cell shape

In addition to the protrusions in the cell membrane and cell size, the overall cell shape was also analyzed. This was accomplished by finding the ratio of the circumference of the cell to its 2D-area. It would be expected that as the number and length of membrane protrusions increase in a cell, the ratio of these two values would increase. Additionally, as the cell shape deviates from a perfect circle, the ratio will go up as well.

The ratio of the circumference to 2D-area was smaller for untransfected cells than that for cells expressing WT or mutant TREK-1 (figure X). This indicates that untransfected cells may have a more normal membrane shape than cells expressing WT and mutant forms of TREK-1. Additionally, the fact that the WT and S46A mutant TREK-1 circumference to 2D-area ratios are nearly identical suggests that although the S46A mutant TREK-1 cells may be smaller, they have a similar cell shape to that of WT TREK-1.

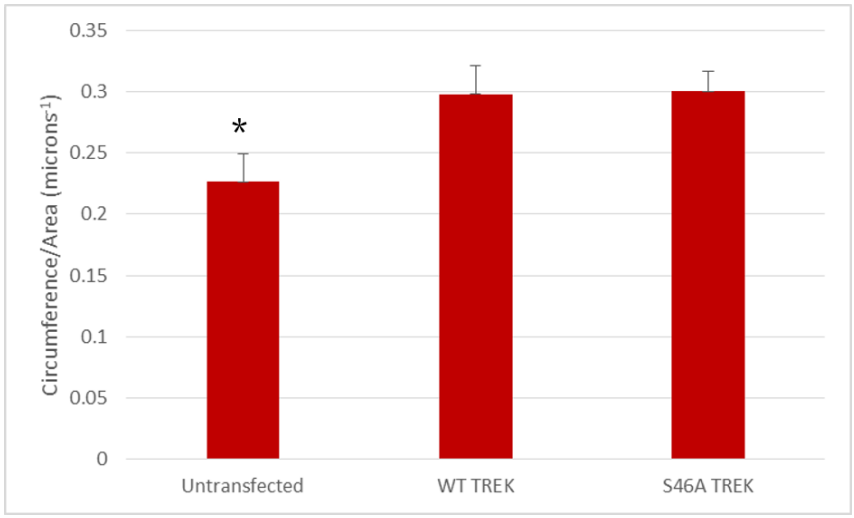

Figure 9: Circumference normalized to the area of cell for untransfected, wild type (WT) and S46A mutant transfected COS M6 cells. P=0.021 between Un and S46A

4. Discussion

Previously, it was reported that CaMKII and TREK-1 were identified as constituents of macromolecular complexes with β_{IV}-spectrin and were reported to play a role in cytoskeleton organization. However, this is the first study to investigate phosphorylation of TREK-1 by CaMKII. The results of the phosphorylation assay indicate that CaMKII phosphorylates TREK-1 in the N-terminus region of the protein. This finding coupled with the preliminary screening of putative CaMKII phosphorylation sites on TREK-1 strongly suggest that phosphorylation occurs at Ser46. However, it would be interesting to analyze the in vivo phosphorylation patterns of TREK-1 using Mass Spectrometry (MS) methods, to both validate the current findings and discover any non-putative phosphorylation sites in TREK-1.

Cell shape, size and structure are dependent upon a large number of factors. Previous studies have shown that TREK-1 induces filopodia-like protrusions in the cell membrane through interaction with actin; however, much is still unknown about how TREK-1 alters the cell membrane and how this morphological change affects the properties and functions of cells. To test the role of TREK-1 and phosphorylation of Ser 46 on cell structure, cells transfected with WT and mutant TREK-1 were analyzed using confocal microscopy. The results of these tests showed that a higher percentage of cells expressing WT TREK exhibited filopodia-like protrusions than that of un-transfected cells or cells expressing TREK-1 with S46A mutation. Cells expressing mutant TREK-1 had a slightly higher percentage of cells exhibiting protrusions than untransfected cells. This trend is also seen in the number of protrusions per cell. This finding reinforces previous studies that have shown the percentage of cells with protrusions increases when TREK-1 is expressed.

The fact that the percentage of cells with protrusions and number of protrusions per cell for S46A mutant TREK-1 was between that of untransfected and cells producing WT TREK-1suggests that the Ser46 site just be one of the factors playing a role in TREK-1's regulation of actin. It was previously shown that the interaction between these two proteins requires phosphorylation at Ser346 and the Glu317 proton sensor in order to elicit actin reorganization, but it is likely that other sites on TREK-1, such as Ser46, also contribute towards TREK-1 induced actin reorganization.

Analysis of the effects WT and mutant TREK-1 have on cell size indicate that Ser46 plays a role in regulating cell size. Both circumference and 2D-area of cells expressing S46A TREK-1 were statistically smaller than that of untransfected cells and cells with WT TREK-1. This combined with the finding that both WT and mutant TREK-1 had similar ratios of circumference to 2D-area suggests that the Ser46 site modulates cell size without having a large impact on the overall cell shape.

These findings are significant because this is the first time that CaMKII has been shown to phosphorylate TREK-1. Understanding the roles of CaMKII is important, especially for understanding cardiac disease where TREK-1 has been shown to be an important ion channel and CaMKII has been linked to diseases such as arrhythmias, hypertrophy, and heart failure. Additionally this research provides further insight into how TREK-1 affects cell structure. TREK-1 mediated actin reorganization could have large effects on the mechanical properties of the cell which in turn could alter TREK-1 current since it is a mechano-sensitive channel. Changes to cell mechanical properties and cell membrane excitability can have large ramifications on cell function at both the cellular and organ level. In cardiomyocytes these changes could alter action potential propagation and cardiac contraction leading to pathologies like arrhythmias or heart failure.

Future research into the mechanism of CaMKII phosphorylation of TREK-1 could use S46A mutant N-terminal TREK for a phosphorylation assay to see if this inhibits phosphorylation, thus pinpointing the exact amino acid of phosphorylation. Additionally, to determine if phosphorylation occurs in vivo, mass spectroscopy or phosphor-specific antibodies could be used to compare phosphorylation levels in TREK-1 between WT cells and cells given a CaMKII inhibitor. Additionally, overexpression of TREK-1 could be induced in cardiomyocytes to determine if actin restructuring occurs. The mechanical properties of cells with reorganized actin due to TREK-1 expression could also be studied by using FEM.

5. Conclusions

Proper ion channel function is essential to human health. Ion channels serve as core constituents of well-defined macromolecular complexes that regulate cell membrane excitability and cell signaling pathways, which affects many different aspects of the cell such as cell structure and function. Importantly, ion channels are heavily regulated by posttranslational modification (e.g. phosphorylation, oxidation), which can have a wide array of effects on how ion channels behave. This research in particular focused on the effects of the two-pore, mechano-sensitive channel TREK-1 and posttranslational modification of this channel on cell structure. It was hypothesized that phosphorylation of TREK-1 by CaMKII would produce phenotypic changes in cell structure. This study used a phosphorylation assay to show a novel CaMKII phosphorylation site on the N-terminus of TREK-1, presumably at Ser46. Studies using immunofluorescent confocal microscopy to examine the shape and size of untransfected cells, cells expressing TREK-1, and cells expressing S46A found that the production of filopodia-like protrusions was slightly upregulated in S46A cells and was largely upregulated in WT TREK-1 expressing cells. These studies also showed that cells expressing the mutant TREK-1 were significantly smaller than both cells without TREK-1 and with WT TREK-1. Lastly, these studies found that although the S46A cells were smaller, they exhibited an overall cell shape similar to that of cells producing WT TREK, which was significantly different from that of the untransfected cells. This research increases our knowledge of how proper cell structure is achieved. These studies also increase our understanding of the roles of TREK-1 and CaMKII at the cellular level and could help us better understand how diseases linked to TREK-1 and CaMKII such as hypertrophy, depression, arrhythmia, and heart failure.

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